

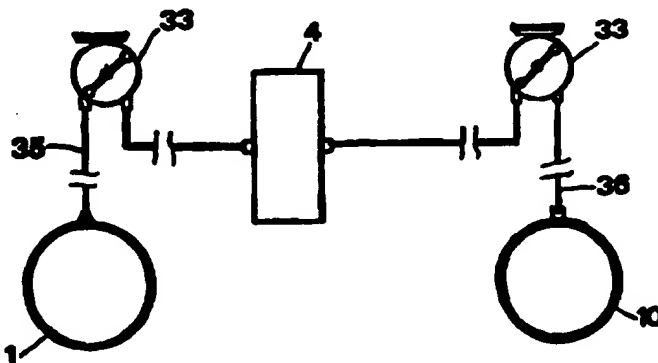


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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**(54) Title:** METHOD FOR THE SPECIFIC IMMUNOADSORPTION OF SELECTED PATHOGENIC FACTORS**(57) Abstract**

An immunopheresis of immunoabsorption method for substracting, according to different cases, specifically selected pathogenic factors (i.e. HIV virus, and/or gp120 antigen, and/or gp120/antibodies anti-gp120 immunocomplexes, and/or TNF- $\alpha$ , and/or interleukins (IL1 $\beta$ , IL4, IL6, IL8, IL10), and/or soluble HLA molecules, and/or gp41 protein, and/or immunocomplexes formed by gp41 and human anti-gp41 antibodies) whose presence is increased during a HIV virus infection and which is directly correlated to a state of acquired immunodeficiency. According to a form of embodiment, a method is provided for the specific immunoabsorption of said pathogenic factors, which comprises drawing blood from a patient affected with a given pathology, adsorbing the pathogenic factors which are present in said blood by using specific ligands (i.e. monoclonal and/or polyclonal anti-gp120 antibodies, and/or anti-HLA antibodies, and/or anti-gp41 antibodies, and/or recombinant CD4 molecules, and/or anti-CD4 antibodies, and/or GNA (Galanthus Nivalis Agglutinin) lectin, and/or a peptide fragment of C1q protein and/or native or recombinant C1q protein, and/or modified or non-modified Staphylococcus aureus A protein, and/or Streptococcus G protein, and/or anti-TNF- $\alpha$  antibodies, and/or anti-interleukins antibodies) in order to obtain blood featuring a low concentration of pathogenic factors.



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**METHOD FOR THE SPECIFIC IMMUNOADSORPTION OF SELECTED  
PATHOGENIC FACTORS.**

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**DEFINITIONS**

10       The following definitions will be adopted throughout the  
present specification:

**VIRUS HIV:**

Any virus belonging to the "Human Immunodeficiency Virus"  
family;

15

**THE PATHOGENIC FACTORS:**

HIV virus, and/or gp120 antigen, and/or gp120/anti-gp120  
immunocomplexes, and/or TNF- $\alpha$ , and/or interleukins (IL1 $\beta$ , IL4,  
IL6, IL8, IL10), and/or soluble HLA molecules, and/or gp41  
protein, and/or immunocomplexes formed by gp41 and anti-gp41  
20       antibodies, and/or human ferritin, and/or bilirubin.

**THE SPECIFIC LIGANDS**

Monoclonal and/or polyclonal anti-gp120 antibodies, and/or  
anti-HLA antibodies, and/or anti-gp41 antibodies, and/or  
recombinant CD4 molecules, and/or anti-CD4 antibodies, and/or  
25       GNA (Galanthus Nivalis Agglutinin) lectin, and/or a peptide  
fragment of Clq protein and/or native or recombinant Clq  
protein, and/or modified or non-modified Staphylococcus aureus  
A protein, and/or Streptococcus G protein, and/or anti-TNF- $\alpha$   
antibodies, and/or anti-interleukins antibodies, and/or anti-  
30       ferritin antibodies, and/or anti-bilirubin antibodies.

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### BACKGROUND ART

The present invention relates to a method for the specific immunoadsorption of pathogenic factors.

### TREATMENT OF AIDS

5 As widely known, HIV virus is a human retrovirus which causes Acquired Immunodeficiency Syndrome (AIDS).

During the acute infection the virus undergoes a massive replication and it is present in patient's peripheral blood (viraemia) for a very short period, followed by a long latency  
10 period during which the virus is present in both peripheral and lymphonodal lymphocytes, but is hardly detectable as free virus in peripheral blood. The latency phase is followed by another viraemic phase characterised by the increase of viral particles and antigens in peripheral blood. This phase is  
15 typically associated to the onset of Acquired Immunodeficiency Syndrome. HIV virus causes immunodeficiency by integration in CD4 cells and causing their death through both direct (cytotoxic) and indirect mechanisms.

The molecular characteristics of the virus, the dynamics  
20 of its integration in the cells, the immune response against it and all the etiopathogenic aspects of the syndrome have constituted the subject-matter of several publications, to which the reader may refer for a complete outline of the state of the art (see, e.g. Fauci A.S. Science 1993, 262:1011-1018).

25 HIV virus produces some viral antigens which are present in serum or in plasma of HIV-positive patients.

A first antigen is constituted by p24, which is typically present during the primary infection (Sidow M. et al. Br. Med. J. 1988 296:238-40), and which is later hardly detectable,  
30 during the syndrome latency phase, due to the production of specific antibodies which mask its recognition by the antibodies which are used for its detection (Lange JMA et al. Br. med. J. 1986 293:1459-62).

The decline of anti-p24 antibodies in serum, and the simultaneous increase of p24 antigen are associated to progression of the syndrome and to clinical deterioration (Allain JP et al. New. Engl. J. Med. 1987 317:1124-21).

5        In vitro HIV-infected cells release gp120 protein, which corresponds to the extracellular unit of the viral envelope.

gp120 may be measured in the serum of HIV-positive patients by using an ELISA test, in which an anti-gp120 antibody is bound to the solid phase and the bound antigen is  
10 detected by a second enzyme-labelled antibody. The gp120 plasmatic concentration ranges from 1 to 100 ng/ml in AIDS patients (Se-Kyung O. et al. J. AIDS: 251-256, 1992). Furthermore, a fraction of plasma gp120 is complexed with anti-gp120 antibodies. This fraction may be detected by a test  
15 which is similar to the preceding one, whereby an antibody specific for human immunoglobulins is used as detector. Use of said test shows that gp120, which circulates in the form of immunocomplexes, is also present before the onset of the Acquired Immunodeficiency Syndrome (SE-Kyung O. et al., J.  
20 AIDS: 251-256, 1992).

It has been demonstrated by several laboratories that native or recombinant gp120 has direct immunosuppressive effects, among which: inhibition of T-proliferative responses, sensibilization to lysis of CD4 cells by means of cytotoxic T  
25 lymphocytes, direct cytotoxicity on nervous cells, aberrant stimulation of chemotaxis of lymphocytes and monocytes, inference of programmed cell death (apoptosis) (Mann DL et al. J. Immunol. 1987, 138:2640-4; Kornfeld H. et al. Nature 1988 335:445-8; Mittler R.S. et al. Science 1989 245:1380-2;  
30 Weinhold KJ et al. J. Immunol. 1989 142:3091-7; Oyaizu N. et al. Proc. Natl. Acad. Sci. USA 1990 87:2379-83; Chirmule N et al. Blood 1990 75:152-9; Brenneman DE et al. Nature 1988 335:639-42; Ameisen J.C. et al. Immunol. Today 1991 12:102-4).

All these effects may result in amplification of T-helper lymphocytes suppression, in destruction of non-infected cells, in neurologic damages and in recruitment of non-infected cells in sites characterised by high virus expression, as spleen of lymphnodes.

All these effects contribute to the pathogenesis of Acquired Immunodeficiency Syndrome. Furthermore, some biological effects of gp120 may be increased or even require the presence of anti-gp120 antibodies cross-linking the protein on the surface of the cells (Mittler R.S. et al. Science 1989: 245:1380-2, CruikShank WW, et al. Biomed. Pharmacother. 1990 44:5-11).

This condition may be detected on most AIDS patients. These immunocomplexes may be pathogenic not only by means of the mechanisms described above, but also through complement activation and a direct proinflammatory effect.

The biological function of gp120 is that of binding to the HIV receptor, the CD4 molecule which is expressed on the membrane of CD4+ helper lymphocytes (Dalglish G. et al. Nature 1984, 312:763-7; Klatzzann D. et al. Nature 1984, 312: 767-8). After binding to CD4, gp120 is shed from the virus, and the transmembrane protein gp41 mediates the fusion between the virus membrane and the cell membrane.

The HIV virus membrane exhibits, in addition to a pair of envelope proteins (gp120 and gp41), other cellular proteins which are probably passively acquired by the cellular membrane during the budding process of the virus (Arthur L. et al. Science 1992, 258:1935-9). Between the cellular proteins which are present on the virus membrane are the antigens of the major histocompatibility complex (HLA), class II and class I. It is possible to capture HIV by means of anti-HLA antibodies, since said antigens are present on the viral membrane in a quantity which is even higher than the quantity of envelope

proteins (Arthur L. et al. Science 1992, 258:1935-9). Some of these antigens, particularly HLA class I, increase in the serum of AIDS patients during the syndrome (Puppo F. et al. J.Laboratory and Clin.Med. 1991 117:91-100), and they may play a directly immunosuppressive role.

It derives therefrom, that selective displacement from serum or plasma of HIV virus, of its mantle antigens (gp120) and of cellular soluble factors increasing during the syndrome, may give a patient a direct benefit by removing some of the immunodeficiency causes.

The therapies which are currently carried out reduce only partially and temporarily the viral load; however, they cannot reduce all those factors which amplify the immunosuppression (circulating antigens and immunocomplexes).

Considerations similar to those which have been disclosed relative to the HIV virus, gp120 antigen and gp120/antibodies anti-gp120 immunocomplexes may be repeated in what concerns Tumor Necrosis Factor  $TNF-\alpha$ , some interleukins ( $IL1\beta$ ,  $IL4$ ,  $IL6$ ,  $IL8$ ,  $IL10$ ), soluble HLA molecules and gp41 protein as well as immunocomplexes formed by gp41 and human anti-gp41 antibodies.

Associated to the progression of Acquired Immunodeficiency Syndrome is an over- or uneven production of  $TNF-\alpha$ , as well as of other inflammatory cytokines (e.g.  $IL1\beta$ ,  $IL4$ ,  $IL6$ ,  $IL8$ ,  $IL10$ ). Hyperproduction of  $TNF-\alpha$  induces septic shock, which constitutes a major death cause inside of intensive care units. A substantial reduction of  $TNF-\alpha$  appears to be of basic importance in the septic shock treatment.

Several compositions are known for in vivo reduction of  $TNF-\alpha$  factor and of other factors cited above (see for instance WO-A1-14464/95, DE-A-4342846, DE-A-4340111, WO-A1-10287/95, EP-A-638556, WO-A2-6077/95, WO-A1-3326/95, EP-A-

636369, WO-A1-514/95, WO-A1-27947/94, EP-A-486809, WO-A1-4675/89, US-A-5364930).

#### TREATMENT OF OTHER PATHOLOGIES

5 Other pathologies characterised by the excessive presence of a given substance in blood are, e.g. Beta Thalassaemia and Cooley's disease, which feature high serum levels of iron.

10 In haemachromatosis of genetic or acquired origin, which is characterised by high levels of ferritin in serum, values ranging from 900 to 6000 micrograms/l are reached, while normal subjects exhibit values comprised between 10 and 200 micrograms/l.

15 This pathology is sometimes treated by means of chelant compositions, which are administered parenterally, and which are able to remove, daily, 10 to 20 mg iron, or through subcutaneous infusions carried out by a low speed portable pump.

Alternately, 500 ml blood are weekly or fortnightly bled.

20 An alternate therapy to bloodletting is constituted by plasmapheresis, which allows a required quantity of patient's blood to be removed and replaced by human albumin or donor's plasma.

25 The therapies involving removal of blood by means of either bloodletting or plasmapheresis reduce the iron level; however, they also induce an undesired removal of all the other substances which are present in blood.

30 Furthermore, the necessity of supplementing the patient's blood with human albumin solutions or with donor's plasma highly increases the patient's risk of contacting viruses such as HBV, HIV, HTLV and others.

Ferritin, which is a protein having a molecular weight of about 46000 Dalton, is constituted by 24 subunits.

Each apoferritin molecule is able of binding



approximately 2000-3000 iron atoms. Plasma iron is stored into two different forms, i.e. ferritin and haemosiderin. About 23% of ferritin total weight is constituted by iron. For the purpose of the present patent application the term "ferritin" shall be understood as including the form haemosiderin too.

The ferritin molecule exhibits iron as oxyde ( $\text{Fe}^{+++}$ ). In order to be transferred to plasma, iron which is contained in ferritin needs to be reduced, i.e. it has to assume the ( $\text{Fe}^{++}$ ) configuration.

Similar considerations may be set out in order to explain the necessity of reducing the level of bilirubin in the therapy of hyperbilirubinaemia or hepatitis, in any form in which they appear.

#### DESCRIPTION OF THE INVENTION

The main purpose of the present invention is to provide for an immunopheresis of immunoadsorption method for substracting, according to different cases, the pathogenic factors (i.e. HIV virus, and/or gp120 antigen, and/or gp120/antibodies anti-gp120 immunocomplexes, and/or  $\text{TNF-}\alpha$ , and/or interleukins ( $\text{IL1}\beta$ , IL4, IL6, IL8, IL10), and/or soluble HLA molecules, and/or gp41 protein, and/or immunocomplexes formed by gp41 and human anti-gp41 antibodies) whose presence is increased during a HIV virus infection and which is directly correlated to a state of acquired immunodeficiency.

Another purpose of the present invention is to provide for a method for substracting human ferritin and bilirubin.

According to a first form of embodiment of the present invention a method is provided for the specific immunoadsorption of said pathogenic factors, which comprises drawing blood from a patient affected with a given pathology, adsorbing the pathogenic factors which are present in said blood by using specific ligands (i.e. Monoclonal and/or

polyclonal anti-gp120 antibodies, and/or anti-HLA antibodies, and/or anti-gp41 antibodies, and/or recombinant CD4 molecules, and/or anti-CD4 antibodies, and/or GNA (Galanthus Nivalis Agglutinin) lectin, and/or a peptide fragment of Clq protein  
5 and/or native or recombinant Clq protein, and/or modified or non-modified Staphylococcus aureus A protein, and/or Streptococcus G protein, and/or anti-TNF- $\alpha$  antibodies, and/or anti-interleukins antibodies, and/or anti-ferritin antibodies, and/or anti-bilirubin antibodies) in order to obtain blood  
10 featuring a low concentration of pathogenic factors.

Advantageously, the ex-vivo immunoadsorption of pathogenic factors from blood also provides for the separation of the corpuscular part of patient's whole blood from plasma, as well as the adsorption of the pathogenic factors by using  
15 the specific ligands in order to obtain plasma featuring a low concentration of pathogenic factors, and mixing such plasma with a suitable quantity of patient's blood corpuscular part.

Immunoadsorption or immunopheresis technique is a specific application of affinity chromatography which is used,  
20 in this case, for subtracting from plasma and/or blood some specific substances which are directly or indirectly responsible of a given disease.

The method according to the present invention is based on the use of specific ligands which are insolublized on an  
25 insoluble solid phase in aqueous solutions, said solid phase being held by a carrier which is permeable to whole blood or plasma.

In the case of treatment of whole blood, venous blood draught from the patient is mixed with an anticoagulant and  
30 sent, by means of a peristaltic pump, to a treatment unit containing the specific ligands, the antibodies and the molecules being insolublized, i.e. linked or otherwise fixed to a suitable carrier through covalent bindings, so as to

enable the specific subtraction of the pathogenic factors by means of a specific immunological antibody-antigen or ligand-receptor reaction.

5 In the specific case of plasma, venous blood draught from the patient is mixed with an anticoagulant and sent to a cell separator, e.g. of a suitable known type, which carries out a separation of the corpuscular part of blood from plasma.

10 Plasma is then sent to a treating module or unit which contains a permeable carrier carrying covalently bound insolublized (in the above described sense) specific ligands, thereby enabling a specific subtraction of pathogenic factors through a specific immunological antibody-antigen or ligand-receptor reaction.

15 Plasma which has been treated in this way may be then mixed with a suitable quantity of blood corpuscular part.

20 According to a further form of embodiment, venous blood collected from a patient and supplemented with an anticoagulant may be preserved within a container, and it undergoes the treatment (i.e. separation of corpuscular part and plasma, adsorption of pathogenic agents and re-mixing with the corpuscular part) only at a second stage (e.g. after several collections, once a predetermined quantity of infected blood has been stored), i.e. before carrying out a massive replacement of patient's blood.

25 According to the invention, the quantity of specific ligands which are insolublized on a suitable carrier may be sufficient for treating all the patient's blood.

#### ILLUSTRATION OF DRAWINGS

30 Other features and advantages of the present invention will become apparent by reading the following description of some ways of carrying out the method according to the invention, with the help of the annexed drawings in which:

- Fig. 1 is a schematical block diagram showing an

apparatus for collecting blood and substracting pathogenic agents from blood;

- Fig. 2 is a schematical illustration of an immunopheresis device for substracting pathogenic agents from extracorporeal plasma according to the present invention;
- Figures 3 and 4 show, each, a schematical representation of covalent (or of other type) bindings between antibody anti-gp120 and/or HLA molecule of Class I and Class II and/or antibody anti-CD4 and/or recombinant CD4 molecules and a solid carrier constituted by a microchannel inside of a microsphere, as well as of an immunological antigen-antibody reaction;
- Fig. 5 is a schematical section view of a treatment module or unit containing specific ligands which are insolublized on a polymeric synthetic matrix;
- Fig. 6 shows a a plan view of a grooved plate belonging to the treatment module of fig. 5;
- Fig. 7 is a section along line IX-IX of fig. 6;
- Fig. 8 is a section view similar to that of fig. 5, showing another form of embodiment of a treatment module;
- Fig. 9 shows a plan view, on a reduced scale, of a shell suitable for housing a module according to fig. 8;
- Fig. 10 shows a reversed section view taken along line VI-VI of fig. 9;
- Fig. 11 is a view from below on a reduced scale of another shell suitable for housing a module according to Fig. 8.

#### DESCRIPTION OF A FORM OF EMBODIMENT OF THE INVENTION

In the drawings identical or similar parts or components bear the same reference signs.

Referring to fig. 1, reference sign 1 generally indicates venous blood draught from a patient infected by HIV virus, reference sign 33 indicates a pair of peristaltic pumps of any

suitable known type, one of which being suitable for feeding the patient's blood towards a treatment module or unit 4 through a pipe 35, while the other one feeds the treated blood 10 into a pipe 36.

5 In figure 2 reference sign 1 indicates venous blood draught from a patient affected with HIV virus, reference sign 2 indicates a centrifugation cell separator, e.g. of any suitable known type, which is able of separating the corpuscular part of blood from plasma, reference sign 3  
10 indicates a pipe for plasma (which contains the pathogenic factors) coming from separator 2 and flowing towards a treatment module or unit 4 containing specific ligands which are insolubilized on a solid carrier. The corpuscular part of blood, separated from plasma inside of separator 2, is sent  
15 through a pipe 5, to a mixing unit 6.

The plasma treated inside module 4 is also sent through a pipe 7 to mixing unit 6, in order to be mixed with the corpuscular part.

As shown in fig. 2, blood 1 to be treated inside of  
20 separator 2, may come from a suitable storing and preserving container 8, inside of which blood 1 might have been stored following to one or more preceding drawings. Furthermore, instead of feeding the plasma treated in the treatment unit or module 4 to mixing unit 6, said plasma may be entirely or  
25 partially preserved inside of a suitable storing container 9 before feeding it to mixing unit 6. This allows a better management of a possible later controlled reinfusion of blood with a low concentration of HIV virus, and/or gp120 antigen, and/or gp120/antibodies anti-gp120 immunocomplexes, and/or  
30 TNF- $\alpha$ , and/or interleukins (IL1 $\beta$ , IL4, IL6, IL8, IL10), and/or soluble HLA molecules, and/or gp41 protein, and/or immunocomplexes formed by gp41 and human anti-gp41 antibodies in the patient.

In the example shown in figures 3 and 4 we schematically represented a pipe passing through a microsphere acting as solid phase 11, which is contained inside of treatment unit 4 and to which are firmly fixed through covalent bindings (12) anti-gp120 antibodies (13), and/or anti-HLA of Class I (14a) and/or of Class II (14b) antibodies, and/or anti-CD4 antibodies (15) suitable for acting against HIV virus (which is represented by an octagone), and/or against gp120 antigen (which is represented by a triangle), and or against gp120/antibodies anti-gp120 immunocomplexes (represented by a rectangle plus a triangle), and/or soluble HLA molecules of Class I and II (which are represented by a smaller blackened rectangle and by a clear one).

Recombinant CD4 molecules, acting on plasmatic gp120 protein and HIV virus, are also fixed to the solid carrier by means of covalent bindings.

According to a general outline, a treatment unit or module 4 may have the configuration which is schematically illustrated in figures 5 to 11. Module 4 is formed by a container, e.g. formed by a pair of external shells 16, 17, which delimit an internal chamber 18 which, in correspondance of shell 16, is joined to the plasma feeding pipe 3, while on the opposite side, in correspondance of shell 17, is joined to evacuation pipe 7. Chamber 18 houses a mechanical entry septum suitable for distributing in the most uniform way the plasma which flows in, an intermediate solid phase 11, which is constituted, for instance, by microspheres or macroporous modules made of 2-hydroxyethylmethacrylate (HEMA) or agarose, cellulose, glass, polyacrylamide, silica, dextran or other synthetic polymers having a diameter of some tens of microns and bearing monoclonal and polyclonal anti-gp120 antibodies, and/or anti-HLA antibodies, and/or anti-CD4 antibodies and/or recombinant CD4 molecules, and a mechanical exit septum 20,

suitable for harvesting the treated plasma and for feeding it into pipe 7.

Solid phase 11 is placed between two microporous filters 21, 22 which are kept separated from each other by means of a  
5 spacing frame 23.

In figure 8 spacing frame 23 extends inwardly between filters 21, 22. Each shell 16, 17 is provided with a respective vent 24, 25 and with peripheral ears 26 cooperating with fastening nuts and bolts.

10 Mechanical septa 19, 20 may have the configuration illustrated in figures 6 and 7, in which the surface directed towards the entry and exit openings is slightly sloping from the centre to the periphery, where some through-holes are provided, while the other surface is either flat or provided  
15 with a series of small concentric ribs 28 which are intersected by radial grooves 29 which, taking into consideration entry septum 19, enhance the plasma distribution over the entire surface of filter 21 and which, taking into consideration exit septum 20, enhance the plasma flow towards  
20 pipe 7.

As previously mentioned, solid phase 11 may be either constituted by microspheres having a microporous structure or by solid microspheres made of polystyrene or divinylbenzene or ethilen-dimethacrylate having a diameter ranging from 5 to 100  
25 micron (and, more precisely, from 20 to 100 micron for plasma and from 200 to 600 micron for whole blood).

Solid phase 11 may also be constituted by one or more porous membranes, and/or by one or more bundles of capillary vessels made of e.g. polyvinylsulfone or tetrafluorethylene,  
30 and/or by spongy or expanded material with open cells.

Anti-gp120 antibodies (13), and/or anti-HLA antibodies of Class I (14a) and/or Class II (14b), and/or recombinant CD4 molecules insolublized on said solid phase 11, come into

contact with virus HIV, and/or antigen gp120, and/or immunocomplexes gp120/antibodies anti-gp120, and/or TNF- $\alpha$ , and/or interleukins (IL1 $\beta$ , IL4, IL6, IL8, IL10), and/or soluble HLA molecules, and/or gp41 protein, and/or  
5 immunocomplexes formed by gp41 and human anti-gp41 antibodies which are present in plasma and they catch them by binding them by means of an immunological antigen-antibody or ligand-receptor reaction.

Treated plasma flows out of treatment unit 4 exhibiting a  
10 lower concentration of virus HIV, and/or antigen gp120, and/or immunocomplexes gp120/antibodies anti-gp120, and/or TNF- $\alpha$ , and/or interleukins (IL1 $\beta$ , IL4, IL6, IL8, IL10), and/or soluble HLA molecules, and/or gp41 protein, and/or immunocomplexes formed by gp41 and human anti-gp41 antibodies,  
15 and it may then be mixed inside of mixing unit 6 with the corpuscular part of blood which is conveyed therein through pipe 5 and which had previously been separated inside of separator 2, in order to re-build whole blood 10.

The invention has previously been described with  
20 reference to an advantageous form of embodiment of the same.

However, it is clear that the invention provides for several forms of embodiment.

As a way of example, the apparatus described above may be used for carrying out a method for subtracting TNF- $\alpha$  factor.

25 In this case solid phase 11, which is represented in fig. 4, may be constituted by a compact, biocompatible and atoxic polymeric resin expressing on its surface active groups suitable for being linked to proteic molecules by means of a covalent binding.

30 By way of example, a first kind of resin may be bound with a specific ligand as polymyxin B, which is an antibiotic able of binding and desactivating endotoxins produced by gram-negative bacteria; a second kind of resin may be bound with a



murine monoclonal antibody (Mab) directed against human cytokin TNF- $\alpha$  or with an either native or recombinant TNF- $\alpha$  receptor.

5 In the following an example is given of a method for covalently binding proteins or molecules to resin Sepharose-4B manufactured by Pharmacia Biotech (Uppsala, Sweden).

#### Method and Materials

10 0,5 g resin in the form of dry powder are weighed for each 5-8 mg protein, e.g. anti-TNF- $\alpha$  antibody, which have to be bound to the solid carrier (1 g resin yields about 3,5 gel after rehydratation).

After rehydratation by means of 1 mM HCl (5 ml for each gram resin), the resin is washed by means of about 200ml acid for each gram resin.

15 The dialyzed proteic solution is mixed, inside of a suitable popypropylene container, with the resin washed with HCl (about 2 ml proteic solution for each ml gel).

The mixture formed by gel and proteic solution is stirred for 4 hours at room temperature.

20 The resin is separated from the buffer solution, which contains the protein which is not covalently bound with the solid carrier, by means of centrifugation at 200-400 g for 10 minutes. The supernatant is harvested by means of aspiration, while the pellet formed by the resin is suspended again with  
25 glycine buffer 0,1 M at pH 8,5 (5 ml buffer for each ml resin).

The resin with glycine buffer is stirred for 18 hours at 4°C.

30 After having separated the resin from the buffer solution by means of centrifugation at 200 g for 10 minutes, the pellet is suspended again with Sodium acetate buffer 0,1 M containing 0,5 M NaCl at pH 4. The resin is washed three times by using the same Sodium acetate buffer. After the third wash the resin

is suspended again with physiological Sodium phosphate buffer and it is washed twice by using the same buffer.

After the second wash by means of the phosphate buffer the resin is suspended again with a phosphate buffer  
5 containing 0,04% Sodium azyde

#### Checking the binding efficiency

The proteic solution harvested after 4 hours incubation with the resin is dialyzed against Sodium carbonate buffer 0,1 M pH 8,5.

10 The proteic concentration of the solution obtained after a 4 hours incubation with resin Sepharose-4B CNBr and of starting proteic solution are determined by using "Protein Assay" kit of Biorad (Richmond, CA).

The protein quantity which is covalently bound with the  
15 resin is determined by using the following formulae:

$$\text{YIELD\%} = 100 - (C2)/C1 \times 100$$

where

C1 = proteic concentration before the covalent binding with resin

20 C2 = supernatant proteic concentration after the first incubation with resin

$$Q = \frac{(C) \times \text{VolP} \times \text{YIELD\%}}{\text{VolR} \times 100}$$

$$\text{VolR} \times 100$$

where

25 Q = protein quantity (mg) bound to the resin

(C) = proteic solution concentration before covalent binding with resin

VolP = proteic solution volume (ml) used for covalent binding with resin

30 VolR = resin volume (ml) obtained at the end of the binding process.

The binding process is then carried out according to the above described method.

According to a further form of embodiment, the apparatus described above may be used for carrying out a method for subtracting ferritin.

5 In this case, solid phase 11 represented in fig. 4 may either be constituted by microporous structured microspheres, or by solid microspheres made of polystyrene or divinylbenzene or polyethylene, or acrylamide, or polyamide, or polyvinylchloride, having a diameter ranging from 40 to 60 micron.

10 The microspheres bear anti-ferritin antibodies which are insolubilized and which come into contact with the ferritin which is present in plasma, and they bind it by means of an immunological antigen-antibody reaction.

15 The plasma, after having contacted the anti-ferritin antibodies which are bound with the solid phase, flows out of the treatment unit exhibiting a low ferritin concentration, and it may then be mixed with the blood corpuscular part, which had previously been separated inside of a suitable separator, in order to re-build whole blood.

20 Similarly, the apparatus may be used for carrying out a method for subtracting bilirubin.

**CLAIMS**

1. Method for substracting in a specific way pathogenic factors from blood or plasma, comprising absortion of said pathogenic factors which are present in blood by means of a specific ligand suitable for capturing said pathogenic factors, said pathogenic factors being respectively constituted by HIV virus, and/or gp120 antigen, and/or gp120/antibodies anti-gp120 immunocomplexes, and/or TNF- $\alpha$ , and/or interleukins (IL1 $\beta$ , IL4, IL6, IL8, IL10), and/or soluble HLA molecules, and/or gp41 protein, and/or immunocomplexes formed by gp41 and human anti-gp41 antibodies.
2. Method according to claim 1, characterised in that said specific ligand is based on monoclonal and/or polyclonal anti-gp120 antibodies, and/or anti-HLA antibodies, and/or anti-gp41 antibodies, and/or recombinant CD4 molecules, and/or anti-CD4 antibodies, and/or GNA (Galanthus Nivalis Agglutinin) lectin, and/or a peptide fragment of Clq protein and/or native or recombinant Clq protein, and/or modified or non-modified Staphylococcus aureus A protein, and/or Streptococcus G protein, and/or anti-TNF- $\alpha$  antibodies, and/or anti-interleukins antibodies.
3. Method for substracting in a specific way pathogenic factors from blood or plasma, comprising absortion of said pathogenic factors which are present in blood by means of a specific ligand suitable for capturing said pathogenic factors, said pathogenic factors being respectively constituted by human ferritin, and/or bilirubin.
4. Method according to claim 3, characterised in that said specific ligand is based on , anti-ferritin antibodies and/or anti-bilirubin antibodies
5. Method according to anyone of claims 2 and 4,

characterised in that said specific ligand is suitable for steadily capturing said pathogenic factors.

- 5 6. Method according to claim 5, characterised in that said specific ligands are steadily bound to an insoluble solid phase in aqueous solutions.
7. Method according to claim 6, as dependent on claims 2 and 5, characterised in that said antibodies and/or recombinant CD4 molecules and/or proteins, and/or lectin, and/or peptidic fragment are bound with the solid phase  
10 by means of covalent bindings.
8. Method according to claim 6 or 7, characterised in that said solid phase comprises a plurality of microspheres.
9. Method according to claim 8, characterised in that said microspheres have a microporous structure.
- 15 10. Method according to anyone of claims 8 and 9, characterised in that said microspheres are made of a material selected from a group including 2-hydroxyethylmethacrylate, polystyrene, divinylbenzene, polyethylene, acrylamide, polyamide, silica, dextran,  
20 agarose, cellulose, glass or other synthetic polymers.
11. Method according to claim 10, characterised in that said microspheres have a diameter ranging from 5 to 1000 micron.
12. Method according to anyone of claims 6 and 7,  
25 characterised in that said solid phase is constituted by at least a porous membrane.
13. Method according to anyone of claims 6 and 7, characterised in that said solid phase is constituted by at least a bundle of microporous capillary vessels.
- 30 14. Method according to claim 13, characterised in that the or each bundle of capillary vessels is made of polyvinylsulfone or tetrafluorethylene.
15. Method according to anyone of claims 6 and 7,

characterised in that said solid phase is constituted by a spongy or expanded material with open cells.

5 16. Method according to anyone of the preceding claims, wherein blood undergoes a treatment for separating the corpuscular part from plasma, wherein only such separated plasma undergoes the adsorption action by means of said specific ligand, so as to obtain plasma exhibiting a low concentration of pathogenic factors, and wherein the plasma treated in this way is then mixed with a suitable  
10 quantity of the corpuscular part of blood which had previously been separated.

17. Method according to claim 14, characterised in that it comprises adding an anticoagulant to blood before separating the corpuscular part from plasma.

15 18. Method according to anyone of claims 14 and 15, characterised in that said separation into corpuscular part and plasma is carried out by centrifugation.

19. Method according to claim 16, characterised in that in comprises preserving at least part of plasma exhibiting a  
20 low concentration of pathogenic agents within a suitable container.

20. Method according to claim 17, characterised in that it comprises preserving at least part of plasma exhibiting a low concentration of pathogenic agents within a suitable  
25 container before mixing it with a suitable quantity of blood corpuscular part.

1/4

Fig. 1

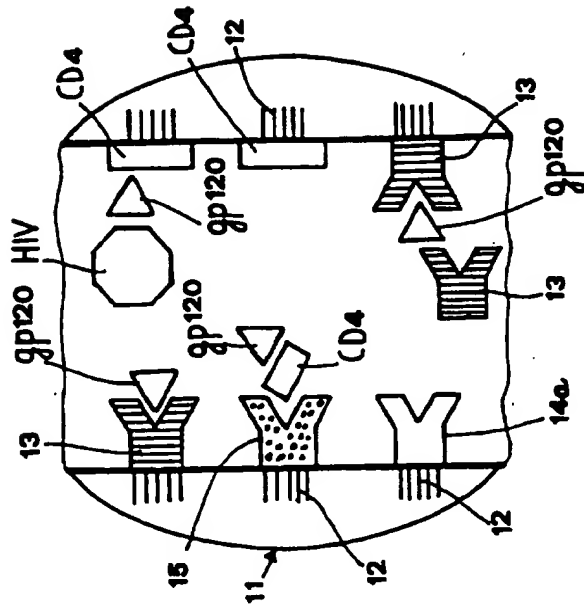
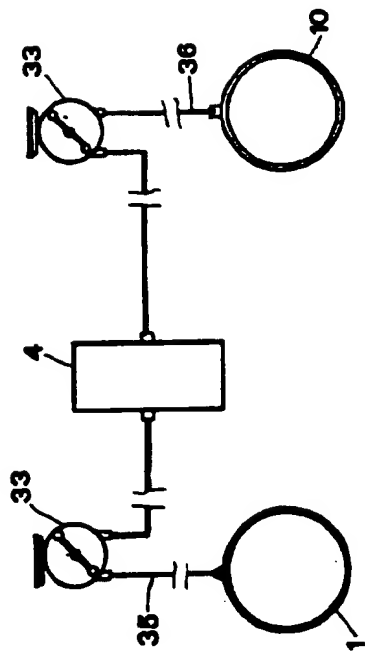


Fig. 3

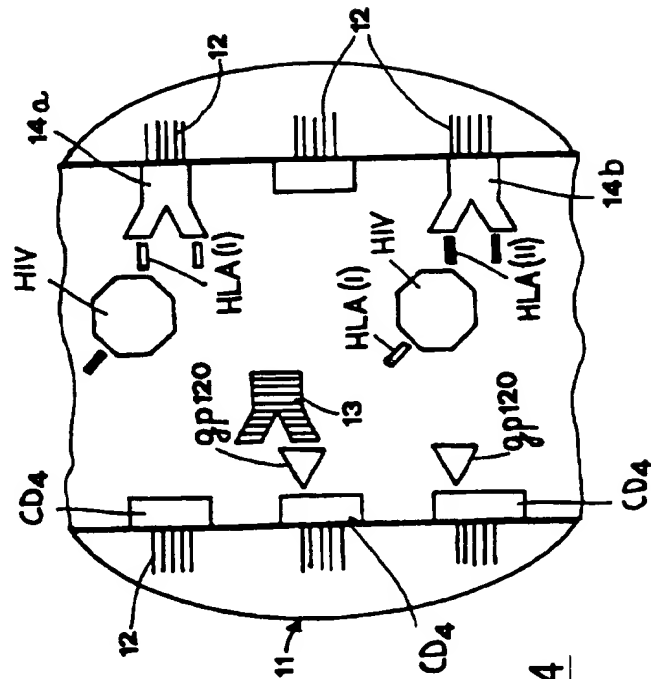
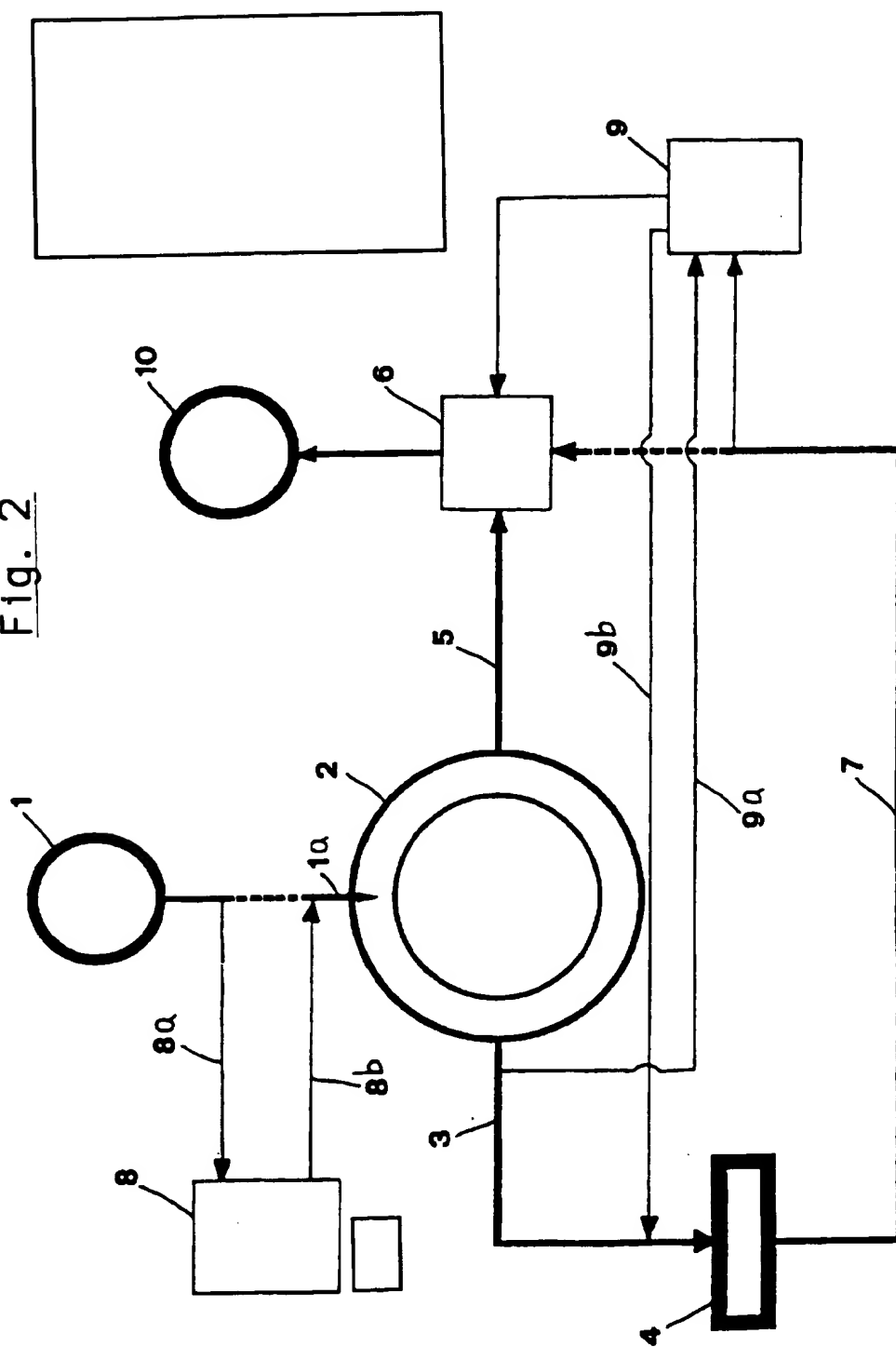


Fig. 4

Fig. 2





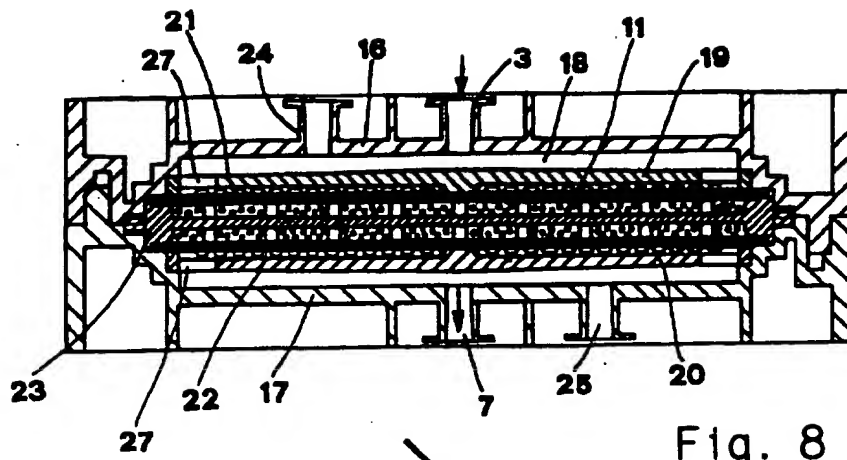


Fig. 8

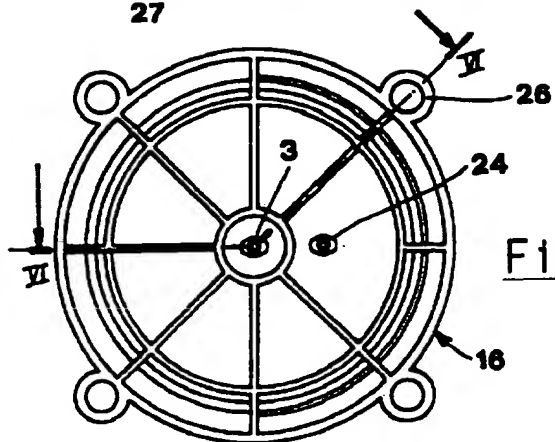


Fig. 9

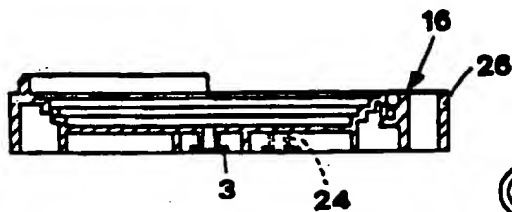


Fig. 11

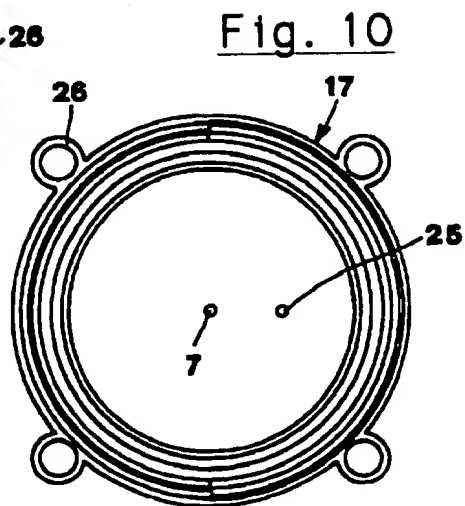


Fig. 10

## INTERNATIONAL SEARCH REPORT

International Application No

PC/IT 95/00206

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K35/14

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	US,A,5 037 649 (BALINT JR JOSEPH P ET AL) 6 August 1991 see the whole document ---	1,2,5-20
X	EP,A,0 592 989 (BRAUN MELSUNGEN AG) 20 April 1994 see the whole document ---	1,2,5-20
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

18 March 1996

Date of mailing of the international search report

26. 03. 96

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## INTERNATIONAL SEARCH REPORT

International Application No

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